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The TLR3 Ligand polyI:C Downregulates Connexin 43 Expression and Function in Astrocytes by a Mechanism Involving the NF- κ B and PI3 Kinase Pathways

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Abstract

Toll-like receptor 3 (TLR3) is a component of the innate immune response that responds to dsRNA viruses and virus replication intermediates. In this study we show that activation of astrocytes with the dsRNA mimetic polyinosinic-cytidylic acid (pI:C) results in loss of expression of connexin43 (Cx43) mRNA and protein while upregulating the expression of the ionotropic P2 receptor P2X₄R. Analysis of the signaling pathways involved failed to demonstrate a role for the p38 MAP kinase, ERK, or JNK signaling pathways whereas an inhibitor of the PI3 kinase/Akt pathway effectively blocked the action of pI:C. Using adenoviral vectors containing a super-repressor of NF- κ B (NF- κ B SR) construct or a dominant negative interferon regulatory factor 3 (dnIRF3) construct showed that inhibition of both transcription factors also blocked the effects of pI:C. To explore the functional consequences of pI:C activation we used a pore-forming assay for P2X₄R activity and a scrape loading assay for gap junction intercellular communication (GJIC). No pore-forming activity consistent with functional P2X₄R expression was detected in either control or activated astrocytes. In contrast, robust Lucifer yellow transfer indicative of GJIC was detected in resting cells that was lost following pI:C activation. The dnIRF3 construct failed to restore GJIC whereas the NF- κ B SR or the NF- κ B inhibitor BAY11-7082 and the PI3K inhibitor LY294002 all significantly reversed the effect of pI:C on GJ connectivity. We conclude that activation of the innate immune response in astrocytes is associated with functional loss of GJIC through a pathway involving NF- κ B and PI3 kinase.

Keywords

CNS glia; innate immunity; gap junctions

INTRODUCTION

The brain is considered to be an immunologically privileged site in which inflammatory/immune responses are tightly regulated. Nevertheless, immunological/inflammatory events do occur in the central nervous system (CNS), often with lethal outcome. Historically, most of

the studies that have addressed CNS-immune interactions have focused on the adaptive immune response, particularly as it relates to autoimmune diseases of myelin. More recently, however, activation of the innate immune response, particularly via toll-like receptors (TLR), has generated much interest (Bsibsi et al., 2006; Carpentier et al., 2005; Farina et al., 2005; Jack et al., 2005; Olson and Miller, 2004; Park et al., 2006; Town et al., 2006). These studies have shown that microglia and astrocytes display different patterns of TLR activation, suggesting distinct roles for these two major populations of glia in shaping the innate, as well as the adaptive, immune response to pathogens in the CNS (Netea et al., 2004).

TLRs constitute a family of type 1 transmembrane proteins that function as recognition receptors for common microbial pathogens. They are characterized by diverse extracellular leucine-rich domains involved in ligand recognition, and a highly conserved cytoplasmic Toll-IL-1R (TIR) domain that triggers a panoply of intracellular signaling pathways (Janeway and Medzhitov, 2002; O'Neill, 2006; Takeda and Akira, 2005). Activation leads to the release of cytokines, chemokines, and costimulatory molecules critical for development of the adaptive immune response and host defense. TLR involved in bacterial pathogen recognition include TLR1 and 2 that recognize triacyl lipopeptides, TLR2 and 6 that recognize diacyl lipopeptides, TLR4 that recognizes lipopolysaccharides of gram-negative bacteria, TLR5 that recognizes flagellin, and TLR9 that recognizes unmethylated DNA sequences found in viral and bacterial DNA. Following viral infections, TLR3 recognizes virus-derived double-stranded RNA (dsRNA), and TLR7 and TLR8 virus-derived single-stranded RNA.

The presence of a highly conserved intracellular domain associated with all TLRs suggests that they share common signaling pathways. Consistent with this is the observation that ligand activation leads to the recruitment of MyD88 and activation of the NF- κ B, JNK, p38 MAP kinase and PI3 kinase signaling cascades (Doyle et al., 2002; O'Neill, 2006; Takeda and Akira, 2005). However, distinct signaling pathways have also been noted, particularly in the production of type 1 interferons (IFN), which are detected following activation of TLR3, 4, 7, 8, and 9. For TLR3 and 4, production of type 1 IFNS is mediated by activation of interferon regulatory factor 3 (IRF3) that occurs through a pathway that is MyD88-independent (Takeda and Akira, 2005).

Because of their role in the innate immune response, TLR have been most extensively characterized in cells of the immune system. However, more recently TLR expression and function has been detected in many different cell types, including astrocytes (Bsibsi et al., 2006; Carpentier et al., 2005; Farina et al., 2005; Jack et al., 2005; Park et al., 2006). Astrocytes are the major glial cell population in the CNS, where they reside ubiquitously in the brain parenchyma as highly ramified cells. They are considered to play an essential role in maintaining the homeostatic environment of the CNS through regulatory effects on key components of every facet of CNS activity (Ransom et al., 2003). Astrocytes also contribute to the development of innate and acquired immune responses by functioning as a major source of chemokines, cytokines and growth factors involved in the initiation and resolution of CNS inflammation (reviewed in John et al., 2005).

Astrocytes are not randomly distributed in the CNS but are organized into nonoverlapping domains (Nedergaard et al., 2003). They are also extensively coupled via two complementary pathways, an intercellular pathway comprised of connexin43 (Cx43) and Cx30 that form gap junction intercellular channels (GJIC), and an extracellular pathway comprised of purinergic P2 receptors that respond to extracellular di- and tri-nucleotides by elevations of intracellular calcium (Scemes, 2000; Nedergaard et al., 2003). GJs allow the intercellular exchange of ions and small molecules, and facilitate long-range signaling thought to be important in the maintenance of brain homeostasis. Connexins present in astrocytes also form GJ channels with connexins present in oligodendrocytes, forming part of a panglial syncytium (Rash et al.,

1997; Nagy et al., 2004, Theis et al., 2005). As such, loss of GJ activity in astrocytes may be expected to significantly affect cell—cell communication in the CNS involving many different cellular functions. In this study, we show that the TLR3 ligand polyinosinic-cytidylic acid (pI:C), which is a potent activator of astrocytes, leads to the loss of Cx43 expression and function via an NF- κ B- and PI3 kinase-dependent pathway. Since GJs form conduits for second messenger systems and metabolites, and contribute to the maintenance of brain homeostasis by facilitating the spatial buffering of K⁺, and the dissipation of potentially toxic mediators such as glutamate (Theis et al., 2004), loss of GJIC in astrocytes may contribute to the damaging effects of inflammation in the CNS.

MATERIALS AND METHODS

Materials

Polyinosinic-cytidylic acid (pI:C, Sigma, St Louis, MO) was diluted in endotoxin free sterile PBS and used at 10 ug/ml unless stated otherwise. Pharmacological inhibitors of signaling pathways included PD098059, a MEK 1 inhibitor; UO126, a dual MEK1 and MEK2 inhibitor; SB202190 and SB203580, p38 MAP kinase inhibitors, LY294002 a PI3kinase inhibitor, and the NF- κ B inhibitor BAY11-7082 (Calbiochem, San Diego, CA). Cells were pretreated with inhibitors at the concentrations shown for 1 h prior to challenge with pI:C.

Astrocyte Cultures

Enriched human fetal brain astrocyte cultures were established from second trimester abortuses as described previously (Lee et al., 1992). All tissue collection was approved by the Institutional Clinical Review Committee of Albert Einstein College of Medicine. Culture medium was Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Bethesda, MD), supplemented with 5% fetal calf serum (FCS) (Gemini, Calabasas, CA) and penicillin (100 U/ml)/streptomycin (100 μ g/ml)/fungizone (0.25 μ g/ml). To obtain cultures essentially free of microglia, cells were plated at 4×10^7 cells per 10 ml medium per T75 cm² tissue culture flask. After 14 days, cultures were shaken to remove microglia and astrocytes subcultured by trypsinization for at least three passages. Culture purity was determined by immunohistochemistry for glial fibrillary acidic protein (GFAP) for astrocytes, (BioGenex, San Ramon, CA), microtubule associated protein-2 for neurons, (Sigma, St Louis, MO) and CD68 for microglia, (DAKO, Carpinteria, CA). Microglia, which are nondividing cells in this system, do not survive this subculturing process and enriched astrocyte cultures typically contain <0.01% microglia contamination. For the studies that employed adenoviral vectors, cells were infected with replication deficient adenoviral constructs encoding either an I κ B α super-repressor or a dominant-negative IRF3 as described previously (Lin et al., 1998; Riveccio et al., 2005). Control preparations included empty vector controls (EV) and a vector expressing wild-type IRF3 (WT) (Lin et al., 1998; Riveccio et al., 2005). Cells were washed with serum-free medium and exposed to different multiplicities of infection (MOI) for 2 h in serum-free medium, washed and incubated in full medium for an additional 48 h prior to pI:C activation.

cDNA Microarray

Three separate cases of human fetal astrocytes plated at 2×10^6 cells/dish were stimulated with 50 ug/ml of pI:C for 6 and 24 h. Cells cultured in medium alone were harvested in parallel. Cell harvest, RNA isolation, and cDNA microarray procedures were performed as described previously (John et al., 2002; Riveccio et al., 2005). Briefly, total astrocyte RNA was extracted using the RNeasy Mini Procedure (Qiagen, Valencia, CA), tested for quality using the Agilent 2100 bioanalyzer, and amplified once using the MessageAmp aRNA kit (Ambion, Austin, TX) before microarray analysis. Total RNA (5 ug) was reverse-transcribed followed by second strand cDNA synthesis. After cDNA purification, amplified antisense RNA (aRNA) was

synthesized by in vitro transcription. Human cDNA arrays were obtained from the Albert Einstein College of Medicine cDNA Microarray Facility (relevant data available from <http://microarray1k.aecom.yu.edu/>). Each slide contained an unbiased, random collection of 28K cDNA probe elements derived from the sequence-verified GEM1 clone set (Incyte Genomics, Palo Alto, CA). Microarray procedures were performed according to protocols provided by the Microarray Facility (<http://microarray1k.aecom.yu.edu/>) and as described (John et al., 2002). Scatter plots were generated using GenePixPro Analysis software (Molecular Devices Corporation, Sunnyvale, CA) from the normalized microarray data.

Real Time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. After treating with DNase I, RNA was reverse transcribed (10 ug total RNA/20 ul) and real time PCR (Q-PCR) performed. Each sample comprised of a 1:1 mixture of diluted (1:100) RT-PCR sample to SYBR green Q-PCR master mix (Applied Biosystems, Foster City, CA) combined with a 1:1 mixture of gene specific forward and reverse primers to the same SYBR green master mix. Plates were processed in an ABI Prism 7000 light cycler (Applied Biosystems, Foster City, CA) using standard cycling conditions. All runs were accompanied by the control genes porphobilinogen deaminase (PBDA) and GAPDH. Samples were normalized using a 2-delta delta Ct based algorithm to give arbitrary units (AU) representing a ratio of experimental to control genes. Primers were selected using www.ensembl.org/ and were Cx43 forward ttccccgatgataaccagaa, reverse gctgtgccacaatggctagt; P2X₄R forward atgatcaacatcggtctgg, reverse aggactatgatgtcacaccacagcac; P2Y₁ forward ttggcgggagatactttcag, reverse gggcatgtcttcactcttgg; IL-8 forward, gcagagggtgttgagaa, reverse ggcattctcactgattcttgg; IP-10 forward, gaatcgaaggccatcaagaa, reverse, gctcccctctggtttaagg. Product size was verified by agarose gel electrophoresis, and melt curve analysis was performed to check primer specificity.

Immunoblotting

Cells were lysed in ice-cold hypotonic lysis buffer (10 mM HEPESpH 7.9, 10mM KCl, 1.5 mM MgCl₂, 50 mM NaF 1mM Na₃VO₄, 1 μM PMSF plus a protease inhibitor cocktail), incubated on ice for 30 min, sonicated and spun at 16,000g for 10 min. Supernatants were separated on 10 or 12% polyacrylamide SDS gels, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% milk in TBS-0.1% Tween-20 for 1 h and incubated overnight with primary antibody (mouse monoclonal anti-Cx43, Clone MAB 3068, Chemicon, 1:500; rabbit anti-P2X₄R, cat #550698, BD Pharmingen, 1:1,000). Membranes were washed with TBS-Tween, incubated for 1 h with goat anti-rabbit horseradish peroxidase-linked secondary antibody in blocking buffer and developed using Supersignal West Pico chemiluminescence ECL kit (Pierce, Rockford, IL), stripped and immunoreacted for tubulin (Sigma clone 2-28-33, 1:1,000) as a loading control, and quantified using Scion NIH Image. Protein content was calculated as a ratio to β-tubulin, and expressed as a percent of the control culture value, which was set at 100%. At least three independent experiments using astrocytes derived from different cases were performed.

Immunohistochemistry

Human fetal astrocytes were plated on MatTek dishes, treated with pI:C for the times indicated, fixed with 4% freshly prepared paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min on ice, washed and permeabilized with PBS containing 10% normal goat serum (NGS) and 0.3% Triton for 10 min on ice. They were then blocked with 10% NGS in PBS for 1 h and incubated with an affinity purified rabbit anti-Cx43 antibody (Zymed-Invitrogen, cat #71-0700), or with an affinity-purified rabbit anti-P2X₄R antibody (BD Pharmingen) diluted 1:100 in 5% milk overnight at 4°. Secondary antibodies were coupled to AlexaFluor 488 or

AlexaFluor 633 (Molecular Probes) diluted 1:100 in PBS containing 1.5% bovine serum albumin (BSA) and 0.1% Triton. Cultures were washed in PBS and stained with an antibody to β -tubulin (Sigma clone 2-28-33) or stained with FITC or rhodamine phalloidin (Molecular Probes, Invitrogen) at 1:20 for 10 min to visualize actin. Nuclei were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes). Control cultures were prepared by omitting the primary antibody. In some experiments, the fluorophores were reversed. Cells were visualized on an Olympus IX70 microscope with a 60 $\times$ N.A. 1.4 infinity-corrected optics, and collected with a Photometrics cooled CCD camera with a KAF 1400 chip using I.P. Lab Spectrum. Cells from at least 3 different brains were examined.

YO-PRO-1 Uptake and Pore Formation

YO-PRO-1 (Molecular Probes) emission was quantified in astrocytes plated in 96-well black-walled, clear bottom plates (Corning International, Corning, NY) using a SpectraMax Gemini spectrofluorimeter running SOFT-maxPRO 3.0 (Molecular Devices, Sunnyvale, CA) with excitation at 491 nm, and emission at 509 nm. All pore formation assays were performed in quadruplicate wells at 37°C using divalent-free solutions to facilitate pore activity. Plates were washed and then incubated with PBS containing 10 μ M YO-PRO-1 (Molecular Probes) with or without ATP or BzATP at dilutions shown. Fluorescence was assayed at times indicated after BzATP or ATP addition and data expressed as a percentage of control, time zero, mean fluorescence intensity (MFI). Astrocytes from two different brains were used for the spectrofluorimetry experiments.

Dye coupling

The scrape loading technique was used to evaluate the degree of gap junctional communication as described (De Pina-Benabou et al., 2001). Astrocytes were plated in 35 mm dishes, grown to confluency, and treated with 10 mg/ml pI:C for 24 h with or without pretreatment for 1 h with specific inhibitors. At the time of testing, cells were washed in divalent ion free PBS pH 7.4, and then incubated at room temperature in PBS containing 0.5 mg/ml Lucifer yellow (LucY) (Sigma). Using a razor blade, three scrapes per dish were made to allow the dye entry and incubated for 10 min at RT. Cells were washed, fixed in 4% paraformaldehyde and photographed using a Leitz DM1RB inverted microscope and a FITC filter using an Olympus DP12 digital camera (Olympus America, Melville, NY). LucY spread was determined by averaging the maximal distances at which LucY fluorescence could be detected using Scion NIH Image software; at least three measurements were obtained along each scrape line in a minimum of three independent experiments.

Statistics

A two-tailed students *T* test at the 95% confidence level was performed using GraphPad Prism software and a *p* value of <0.05 was considered significant.

RESULTS

pI:C Differentially Regulates Cx43 and P2X₄R Expression in Human Astrocytes in Culture

To determine the pattern of gene expression induced by the dsRNA analog pI:C in human fetal astrocytes, we first performed a cDNA microarray analysis using a human 28K chip and cells activated for 6 or 24 h. The advantage of this approach is that both positively and negatively regulated genes can be readily identified using the same screen. A representative experiment for gene expression in astrocytes following activation with 10 μ g/ml pI:C for 24 h is shown in Fig. 1A. Data are presented as a scatter plot of the mean fluorescence intensities for each gene on the X-axis, which is proportional to mRNA expression levels, graphed against the median fold change from control untreated cells of all the genes represented on the array induced by

pI:C on the Y-axis with each data point above 1 representing genes with increased expression and each data point below 1 representing genes with decreased expression following activation with pI:C. The data showed that pI:C transcriptionally activated a broad spectrum of genes, which included inflammatory mediators such as IL-8 (arrow, Fig. 1A) (Rivieccio et al., in press), consistent with other studies (Bowman et al., 2003; Carpentier et al., 2005; Farina et al., 2005; Jack et al., 2005; Park et al., 2006). However, the data also showed that pI:C potentially downregulated a number of genes, including several that were expressed at high levels in nonactivated control astrocytes. Examination of the spots included in this group showed that one of the markedly downregulated genes was Cx43 (arrow, Fig. 1A). Analysis of the data across multiple experiments ($n = 3$) showed ratios of pI:C to control at 6 h for Cx43 of 0.5, 0.3, and 0.4; and for IL-8 of 49.9, and 19.2 (one spot damaged); and at 24 h for Cx43 of 0.15, 0.22, and 0.04, and for IL-8 of 326, 317, and 114.

Astrocytes are extensively coupled by two interconnected pathways, GJIC and purinergic P2 receptors. Therefore, we also probed the microarray data for effects of pI:C on other GJ proteins and P2 receptors. No detectable signal was found for Cx30, the other major astrocyte GJ protein, which was not unexpected since this connexin is not found in fetal tissues (Kunzelman et al., 1999), and the signal for Cx26 was present only at very low levels (data not shown). Relatively few P2 receptors were present on our cDNA gene chip, with the exception of the ionotropic P2X₄ receptor, which we found to be positively regulated in these cultures.

To validate the microarray data for Cx43 and P2X₄R, expression of these genes was examined using real-time PCR (Q-PCR), western blotting and IHC. For the Q-PCR, cells were activated with 10 mg/ml pI:C and RNA samples prepared at 3, 6, and 24 h poststimulation. Control samples were harvested at the 24 h time-point. Q-PCR for the chemokines IP-10 and IL-8 were included as genes known to be positively regulated in astrocytes by TLR3 (Farina et al., 2005; Jack et al., 2005; Park et al., 2006), as well as for P2Y₁, a member of the meta-botropic P2 receptor family. Using PBDA as the control reference gene and the $2^{-\Delta C_T}$ comparative method of analysis (see methods), both IP-10 and IL-8 showed robust induction by pI:C in astrocytes with values increasing with time (Fig. 1B). The relatively late activation of IL-8 is consistent with the delayed activation of NF- κ B noted following TRIF-dependent signaling (Doyle et al., 2002; Sen and Sarkar, 2005). A similar, albeit less robust, activation of the P2X₄R was also noted. In contrast, pI:C led to the rapid loss of mRNA for Cx43 over the same time-period. The results for P2Y₁ were variable, but showed no significant difference from control cells (data not shown). Pretreatment of the cells for 30 min with 10 μ M of cyclohexamide prior to activation with pI:C for 3 h showed that loss of Cx43 and induction of P2X₄R mRNA was a direct effect of pI:C and not a secondary response to other proteins induced by pI:C activation (data not shown). Thus, using Q-PCR the data for both Cx43 and P2X₄R were consistent with that found on the microarrays.

Western blots of pI:C-treated cultures showed similar changes for protein expression. For Cx43 a reduction in total protein was already evident at 3 h poststimulation, and was considerably reduced by 24 h (Fig. 2A). In contrast, prominent induction of P2X₄R was only noted 24 h after pI:C stimulation. These effects of pI:C were dose-dependent (Fig. 2B).

Immunohistochemical analysis of the distribution of Cx43 in astrocytes showed dense foci of Cx43 immunoreactivity at sites of fine cytoplasmic bridges between the cells, consistent with the formation of GJ plaques (Fig. 2C). Following activation with 10 mg/ml pI:C for 24 hr, many astrocytes in culture changed from a predominantly flat polygonal cell to a more process-bearing cell. In these cultures, few GJ plaques were detected (Fig. 2C). Staining for P2X₄R in control cultures gave a fine punctate pattern of reactivity, with no obvious membrane staining. In pI:C-treated cultures immunoreactivity for P2X₄R was increased, but the staining pattern was unchanged (Fig. 2C).

pI:C-Induced Loss of Cx43 is Mediated by the PI3kinase Pathway But Not by the p38 MAP Kinase, JNK or ERK Signaling Pathways in Astrocytes

Following interaction with ligand, signaling via the TLR3 receptor is known to proceed via the upstream signaling complex consisting of the Toll/IL-1R domain containing-adaptor inducing IFN β (TRIF) and the TANK-binding kinase (TBK)1, resulting in the subsequent activation of multiple downstream kinases such as p38 MAP kinase, extracellular signal-related kinase (ERK) 1 and 2, JNK and PI3K, as well as the transcription factors NF- κ B and IRF-3 (Sen and Sarkar, 2005).

To test for a role for these kinases in mediating pI:C-induced loss of Cx43 expression, specific pharmacological inhibitors were used in conjunction with western blotting. Inhibitors of MEK1/2, ERK, and JNK were either without effect, or showed only partial inhibition at higher dose levels (Fig. 3A). Similarly, inhibitors of p38 MAP kinase (SB203580 and SB202190) blocked pI:C activation of astrocytes but only at concentrations at or above 20 μ M. The requirement for this dose of SB203580 was of concern, since questions have been raised regarding the specificity of this inhibitor at this dose. To explore further a possible role for p38 MAP kinase in pI:C-induced loss of Cx43, these assays were repeated using adenoviral vectors incorporating a dominant negative form of either the α or β isotype of p38 MAP kinase (kindly supplied by Dr. Amit Verma, Albert Einstein College of Medicine). However, neither of these vectors resulted in a reversal of the effect of pI:C, even though analysis of p38 expression showed that the vectors were appropriately expressed (data not shown).

PI3K catalyses the production of phosphatidyl 3,4,5-tri-phosphate, resulting in the recruitment of signaling proteins that include the serine-threonine kinase Akt. To define the role of this signaling pathway in pI:C-induced loss of Cx43, the specific inhibitor Ly294002 was used. As shown in Fig. 3B, this inhibitor significantly blocked pI:C-induced loss of Cx43 protein as well as induction of P2X₄R expression in a dose-dependent fashion.

Adenoviral Vectors Expressing an I κ B α Super-Repressor and a dn-IRF3 Block the Effects of pI:C on Cx43 and P2X₄R Protein Expression

NF- κ B is an important transcription factor for many cytokines, chemokines, and effector mechanisms associated with inflammation (Sen and Sarkar, 2005), whereas IRF-3 is a required transcription factor for many antiviral proteins such as the type 1 interferons following activation through TLR3 (Takeda and Akira, 2005). Analysis of genes activated by pI:C in human fetal astrocytes indicated that both pathways are prominent targets of TLR3 signaling in astrocytes (Rivieccio et al., manuscript in press). To test for their role in the pI:C-dependent regulation of Cx43 and P2X₄R gene expression in these cells, we used adenoviral vectors encoding the proteolysis resistant I κ B α super-repressor (S32,S36E, AdI κ BaSR kindly provided by Dr. Mark J. Czaja, Albert Einstein College of Medicine) or a dominant negative form of IRF3 (dnIRF3) in which the N-terminal DNA-binding domain has been deleted (Lin et al., 1998; Rivieccio et al., 2005). Cells were incubated for 2 days with three different concentrations (1×10^3 , 3×10^3 , and 10×10^3 particles/ml) of each of the adenoviral constructs, along with appropriate controls, activated with pI:C at 10 μ g/ml for 24 h, and levels of Cx43 and P2X₄R determined by western blotting. As shown in Fig. 4A, the I κ B α super-repressor dose-dependently inhibited both pI:C-induced loss of Cx43 and induction of P2X₄R. In contrast, exposure of cultures to either adenoviral empty vectors (Ad-EV) alone, or in combination with pI:C, had no effect (data for only two doses shown). Combined data for astrocytes derived from three different cases are shown in the graph (Fig. 4B).

To substantiate further the role of the NF- κ B signaling pathway, we applied BAY 11-7082, an inhibitor of NF- κ B that prevents the phosphorylation of the inhibitory protein I κ B and the

release of bound NF- κ B components. This inhibitor also reversed the downregulatory effect of pI:C for Cx43 expression (Fig. 4C).

The experiments were then repeated using an adenoviral vector containing the dnIRF3 (Fig. 5A). In agreement with the effects of the I κ B α super-repressor, dnIRF3 constructs also dose-dependently inhibited the pI:C-induced loss of Cx43 and induction of P2X₄R, whereas empty vectors (EV) or adenoviral vectors expressing WT-IRF3 (WT) were without effect. Combined data for astrocytes derived from three different cases are presented graphically (Fig. 5B).

Functional Studies of the P2X₄Rin pI:C-Treated Astrocytes

We then sought to determine whether the pI:C-induced changes in expression of the P2X₄R and Cx43 had functional significance. First, we used a pore-forming assay for P2X₄R. Following ligand binding both the P2X₇R and P2X₄R are known to form pores in the cell membrane that allow passage of molecules up to 900 Da (North and Surprenant, 2000). To test for pore formation, we measured uptake of the fluorescent dye YoPro-1 following stimulation of control and pI:C-activated cells with varying concentrations of the P2 receptor agonists ATP and BzATP. Control, untreated cells were generally unresponsive to ligand binding with only 300 μ M BzATP inducing a low level, time-dependent uptake of YoPro-1 (Fig. 6A). Following activation with pI:C, astrocytes showed an enhanced time-dependent uptake of YoPro-1, but again this only occurred following stimulation with 300 μ M BzATP, with other concentrations of BzATP or ATP having no effect (Fig. 6B). This agonist profile is characteristic of the P2X₇R rather than the P2X₄R, suggesting that the P2X₄R is not functionally displayed on the cell surface in pI:C-stimulated astrocytes.

Functional Studies of GJIC Communication in pI:C-Treated Astrocytes

Next we determined whether the pI:C mediated down-regulation of Cx43 was associated with functional loss of GJs in astrocytes, and if so which signaling pathway(s) were involved. To do this we used a scrape-loading paradigm and the GJ permeant dye Lucifer yellow. Cells in control cultures were extensively coupled, as determined by the spread of the fluorescent dye from the edge of the cut (Fig. 7A). Activation with 10 mg/ml pI:C for 24 h resulted in marked loss of dye coupling (29% of control values). Use of the dnIRF3 construct failed to restore dye coupling (35% of control values), whereas the I κ B α -SR resulted in significant recovery (71% of control values) (Fig. 7B).

The observation that dnIRF3 blocked pI:C-dependent loss of Cx43 but did not restore function raised the question as to whether in these cells Cx43 protein failed to appropriately target the cell membrane. To test for this we performed IHC for Cx43 in cells treated with 10 mg/ml pI:C without and with pretreatment with adenoviral vectors containing the I κ B α super-repressor or the dn-IRF3 for 48 h. In cells treated with I κ B α -SR, IHC showed prominent staining at membrane sites associated with fine cytoplasmic bridges that formed between the cells, as noted in the controls (Supplementary Figs. 1A,B). However, in cells pretreated with dnIRF3 and then activated with pI:C, large accumulations of Cx43 immunoreactivity were noted at the cell membrane, but which failed to make appropriate contacts with adjacent cells (Supplementary Fig. 1D). These data show that recovery of protein levels, as was noted with the dnIRF3 construct, does not necessarily equate with functional recovery of GJIC.

The scrape-loading assays were then repeated using the PI3K inhibitor LY294002 and the pharmacological inhibitor of NF- κ B BAY11-7082. The data showed that both LY294002 and BAY11-7082 significantly restored GJIC in pI:C-treated astrocytes, and that a combination of LY294002 and BAY11-7082, both at 5 μ M, fully reversed the down-regulatory effect of pI:C on Cx43 expression and function in human fetal astrocytes in culture.

DISCUSSION

In this study we show that activation of astrocytes with pI:C leads to rapid loss of Cx43 that correlated with loss of GJIC in these cells. pI:C is a mimic for the TLR3 ligand dsRNA, which occurs in some RNA viruses, as well as in intermediates that form during viral replication. TLR3 activation may also occur in response to cellular RNA that is released as a result of tissue damage occurring within the CNS (Kariko et al., 2004). These data, in conjunction with the pattern of gene activation detected by microarray (see Fig. 1) lend further support to the observation that ligands for TLR3 potently activate human astrocytes in vitro and in vivo (Jack et al., 2005; Farina et al., 2005; Town et al., 2006), and strongly implicate astrocytes as a major component of the innate immune response in the CNS, particularly as it relates to viral infections. However, whether this response is protective or deleterious for CNS function may, like LPS, be critically dependent upon the specific viral infection involved and the genetic make-up of the host (Wang et al., 2004; Bsibsi et al., 2006; Town et al., 2006).

Loss of Cx43 activity was associated with a reciprocal increase in mRNA and protein for the ionotropic P2X₄R. However, this did not correlate with any indication of increased functional activity for this receptor. This result is consistent with our previous studies that noted an increase only in P2X₇R-dependent responses in cytokine-activated astrocytes (Narcisse et al., 2005). These data suggest that additional signals are required for functional expression of the P2X₄R at the cell membrane, and posttranslational modifications, such as phosphorylation events, may control this activity as has been noted for P2X₇R function (Adinolfi et al., 2003).

The observation that Cx43 is a target of pI:C-induced signaling in astrocytes is in agreement with other studies that have shown loss of Cx43 in response to inflammatory mediators in astrocytes as well as other cell types. Thus, both we and others have shown that soluble products of microglia/macrophages such as the cytokines IL-1 and TNF can lead to loss of Cx43-dependent GJIC (Hinkerohe et al., 2005; John et al., 1999; Meme et al., 2006; Rouach et al., 2002). Decreased Cx43 has also been noted in vivo in mice sensitized to develop experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (Brand-Schieber et al., 2005). In these mice, loss of Cx43 was only detected in areas of inflammation in the spinal cord, whereas adjacent white matter tracts showed normal Cx43 immunoreactivity. Analysis of Cx43 mRNA and protein expression levels in the spinal cord confirmed the immunohistochemical data. Alterations in Cx43 expression have also been noted in a number of different human CNS pathologies. For example, in gliomas a neoplastic phenotype is associated with reduced Cx43 expression (Naus et al., 2001). Reduced Cx43 expression has also been noted following traumatic and ischemic injury to the CNS, whereas elevated expression has been noted in lesions associated with Alzheimer's and Parkinson's diseases (reviewed in Rouach et al., 2002).

At present, the functional consequences of loss of GJIC at sites of inflammation are not fully understood. In the CNS, astrocytes are the major population of cells coupled by GJs, with functional activity that persists throughout adulthood (Rouach et al., 2002). Cx43 GJs are particularly abundant on astrocytic processes en-sheathing chemical synapses and nodes of Ranvier, as well as on astrocytic endfeet surrounding the vasculature. Mice in which the gene for Cx43 has been inactivated, die at birth due to cardiac dysfunction, but Cx43-null heterozygotes (Cx43 +/-) are viable, fertile and show no gross functional or structural abnormalities. Cx43 +/- mice subjected to middle cerebral artery occlusion (MCAO) show enhanced stroke volume and apoptosis of neurons compared to WT controls, supporting the conclusion that astrocytes play a critical role in neuroprotection (Naus et al., 2001; Nakase et al., 2003). However, in the Cx43 +/- mice, Cx43 expression is compromised in many cell types and organs other than astrocytes, making it difficult to determine the role that astrocytic GJs play in regulating and/or contributing to CNS pathology in vivo. To circumvent this problem,

Willecke and colleagues have developed an astrocyte-targeted Cx43 knock-out using a floxed Cx43 and the hGFAP_{Cre} mouse (Theis et al., 2003) [Cx43(fl/fl): hGFAP_{Cre}]. In this mouse, MCAO also resulted in an enhanced stroke volume, increased neuronal apoptosis, as well as accelerated spreading depression (Nakase et al., 2004). Cx43 in astrocytes also forms heterotypic GJs with connexins present in oligodendrocytes, suggesting that loss of Cx43 function may also affect other populations of glia as well as myelin (Kamasawa et al., 2005). In this regard, it is of interest to note that in oculodentodigital dysplasia (ODDD), a rare autosomal recessive disorder associated with polymorphisms in the Cx43 gene, neurologic signs are common, and include dysarthria, neurogenic bladder disturbances, spastic paraparesis, ataxia, anterior tibial muscle weakness, and seizures (Loddenkemper et al., 2002). Brain magnetic resonance imaging studies of ODDD patients show diffuse bilateral abnormalities in the subcortical cerebral white matter associated with a slowly progressive leukodystrophy (Paznekas et al., 2003). This evidence of white matter disease in ODDD is intriguing, since it has been proposed that coupling of astrocytes with oligodendrocytes in the CNS would allow astrocytes to provide metabolic and ionic support to the several myelinating segments formed by each oligodendrocyte (Nagy et al., 2004; Kamasawa et al., 2005).

The signaling pathway activated by TLR3 differs from other TLR family members in that it proceeds almost exclusively via the adaptor protein Toll/IL-1R domain-containing adaptor inducing IFN β (TRIF), independently of the adaptor protein MyD88. Signaling then bifurcates downstream of TRIF such that TRIF-TRAF6 interactions lead to delayed activation of NF- κ B, and TRIF/TBK1 interactions to activation of IRF3 (reviewed in O'Neill et al., 2005; Sen and Sarkar, 2005; Takeda and Akira, 2005). Nuclear translocation of NF- κ B is required for the activation of a wide range of genes that play a pivotal role in inflammatory and immune-mediated events such as cytokines and chemokines, whereas phosphorylation, dimerization and nuclear translocation of IRF3 is required for production of IFN-related genes. Analysis of the signaling pathways involved in loss of Cx43 in astrocytes showed that inhibition of the NF- κ B pathway restored both Cx43 protein and GJ function, whereas inhibition of the IRF3 signaling pathway restored Cx43 protein but not GJ function. The data further showed that, whereas various components of the MKK pathways were not involved in pI:C-induced down-regulation of Cx43, the PI3 kinase inhibitor Ly294002 significantly restored GJIC in these cultures of primary human fetal astrocytes. Previous studies have shown that following ligand interaction with TLR3, PI3K positively regulates IRF3-dependent IFN β induction, but negatively regulates TRIF/TRAF6-dependent NF- κ B signaling (Sen and Sarkar, 2005). Recent studies in a human astrocyte cell line, CRT-mG, would be consistent with this role for PI3K since in those studies pretreatment with LY294002 inhibited TLR3-induced IP10 (an IRF3-dependent gene) but potentiated IL-8 (an NF- κ B-dependent gene) expression (Park et al., 2006). Our data indicating that inhibitors of both NF- κ B activation and PI3K blocked TLR3-mediated down-regulation of Cx43 is at variance with this signaling pathway. However, NF- κ B is a highly complex transcription factor that requires several steps for full activation (Perkins and Gilmore, 2006), and PI3K may also lead to activation of NF- κ B through relieving transcriptional repression of the p65 subunit of NF- κ B. PI3K is also critical for promoting the nuclear translocation and DNA binding of the p50 subunit of NF- κ B (Choi and Jeong, 2005). Thus how PI3K signaling intersects with the NF- κ B pathway leading to alterations in Cx43 gene expression following pI:C activation of astrocytes remains unclear at the present time, and will require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Adinolfi E, Kim M, Young MT, Di Virgilio F, Surprenant A. Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. *J Biol Chem* 2003;278:37344–37351. [PubMed: 12869560]
- Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. *Glia* 2003;43:281–291. [PubMed: 12898707]
- Brand-Schieber E, Werner P, Jacobas DA, Jacobas S, Beelitz M, Lowery SL, Spray DC, Scemes E. Connexin43, the major gap junction protein of astrocytes, is down-regulated in inflamed white matter in an animal model of multiple sclerosis. *J Neurosci Res* 2005;80:798–808. [PubMed: 15898103]
- Bsibsi M, Persoon-Deen C, Verwer RW, Meeuwse S, Ravid R, Van Noort JM. Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. *Glia* 2006;53:688–695. [PubMed: 16482523]
- Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ, Miller SD. Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia* 2005;49:360–374. [PubMed: 15538753]
- Choi YS, Jeong S. PI3-kinase and PDK-1 regulate HDAC1-mediated transcriptional repression of transcription factor NF-KB. *Mol Cells* 2005;20:241–246. [PubMed: 16267399]
- De Pina-Benabou MH, Srinivas M, Spray DC, Scemes E. Calmodulin kinase pathway mediates the K⁺-induced increase in Gap junctional communication between mouse spinal cord astrocytes. *J Neurosci* 2001;21:6635–6643. [PubMed: 11517253]
- Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R, Cheng G. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 2002;17:251–263. [PubMed: 12354379]
- Farina C, Krumbholz M, Giese T, Hartmann G, Aloisi F, Meinl E. Preferential expression and function of Toll-like receptor 3 in human astrocytes. *J Neuroimmunol* 2005;159:12–19. [PubMed: 15652398]
- Hinkerohe D, Smikalla D, Haghikia A, Heupel K, Haase CG, Dermietzel R, Faustmann PM. Effects of cytokines on microglial phenotypes and astroglial coupling in an inflammatory coculture model. *Glia* 2005;52:85–97. [PubMed: 15920725]
- Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, Shapiro A, Antel JP. TLR signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol* 2005;175:4320–4330. [PubMed: 16177072]
- Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216. [PubMed: 11861602]
- John GR, Lee SC, Song X, Rivieccio M, Brosnan CF. IL-1-regulated responses in astrocytes: Relevance to injury and recovery. *Glia* 2005;49:161–176. [PubMed: 15472994]
- John GR, Scemes E, Suadicani SO, Liu JS, Charles PC, Lee SC, Spray DC, Brosnan CF. IL-1 β differentially regulates calcium wave propagation between primary human fetal astrocytes via pathways involving P2 receptors and gap junction channels. *Proc Natl Acad Sci USA* 1999;96:11613–11618. [PubMed: 10500225]
- John GR, Shankar SL, Shafit-Zagardo B, Massimi A, Lee SC, Raine CS, Brosnan CF. Multiple sclerosis: Re-expression of a developmental pathway that restricts oligodendrocyte maturation. *Nat Med* 2002;8:1115–1121. [PubMed: 12357247]
- Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KG, Nagy JI, Rash JE. Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt-Lanterman incisures: Implications for ionic homeostasis and potassium siphoning. *Neuroscience* 2005;136:65–86. [PubMed: 16203097]

- Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem* 2004;279:12542–12550. [PubMed: 14729660]
- Kunzelmann P, Schroder W, Traub O, Steinhauser C, Dermietzel R, Willecke K. Late onset and increasing expression of the gap junction protein connexin30 in adult murine brain and long-term cultured astrocytes. *Glia* 1999;25:111–119. [PubMed: 9890626]
- Lee SC, Liu W, Brosnan CF, Dickson DW. Characterization of primary human fetal dissociated central nervous system cultures with an emphasis on microglia. *Lab Invest* 1992;67:465–476. [PubMed: 1359193]
- Lin R, Heylbroeck C, Pitha PM, Hiscott J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* 1998;18:2986–2996. [PubMed: 9566918]
- Loddenkemper T, Grote K, Evers S, Oelerich M, Stogbauer F. Neurological manifestations of the oculodentodigital dysplasia syndrome. *J Neurol* 2002;249:584–595. [PubMed: 12021949]
- Meme W, Calvo CF, Froger N, Ezan P, Amigou E, Koulakoff A, Giaume C. Proinflammatory cytokines released from microglia inhibit gap junctions in astrocytes: Potentiation by β -amyloid. *FASEB J* 2006;20:494–496. [PubMed: 16423877]
- Nagy JI, Dudek FE, Rash JE. Update on connexins and gap junctions in neurons and glia in the mammalian nervous system. *Brain Res Brain Res Rev* 2004;47:191–215. [PubMed: 15572172]
- Nakase T, Fushiki S, Sohl G, Theis M, Willecke K, Naus CC. Neuroprotective role of astrocytic gap junctions in ischemic stroke. *Cell Commun Adhes* 2003;10:413–417. [PubMed: 14681050]
- Nakase T, Sohl G, Theis M, Willecke K, Naus CC. Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes. *Am J Pathol* 2004;164:2067–2075. [PubMed: 15161641]
- Narcisse L, Scemes E, Zhao Y, Lee SC, Brosnan CF. The cytokine IL-1 β transiently enhances P2X7 receptor expression and function in human astrocytes. *Glia* 2005;49:245–258. [PubMed: 15472991]
- Naus CC, Ozog MA, Bechberger JF, Nakase T. A neuroprotective role for gap junctions. *Cell Commun Adhes* 2001;8:325–328. [PubMed: 12064612]
- Nedergaard M, Ransom B, Goldman SA. New roles for astrocytes: Redefining the functional architecture of the brain. *Trends Neurosci* 2003;26:523–530. [PubMed: 14522144]
- Netea MG, van der Graaf C, Van der Meer JW, Kullberg BJ. Toll-like receptors and the host defense against microbial pathogens: Bringing specificity to the innate-immune system. *J Leukoc Biol* 2004;75:749–755. [PubMed: 15075354]
- North RA, Surprenant A. Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol* 2000;40:563–580. [PubMed: 10836147]
- Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol* 2004;173:3916–3924. [PubMed: 15356140]
- O'Neill LA. How toll-like receptors signal: What we know and what we don't know. *Curr Opin Immunol* 2006;18:3–9. [PubMed: 16343886]
- Park C, Lee S, Cho IH, Lee HK, Kim D, Choi SY, Oh SB, Park K, Kim JS, Lee SJ. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: Differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. *Glia* 2006;53:248–256. [PubMed: 16265667]
- Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE, Innis JW, Dinulos MB, Christian C, Hannibal MC, Jabs EW. Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet* 2003;72:408–418. [PubMed: 12457340]
- Perkins ND, Gilmore TD. Good cop, bad cop: the different faces of NF-KB. *Cell Death Differ* 2006;13:759–772. [PubMed: 16410803]2006
- Ransom B, Behar T, Nedergaard M. New roles for astrocytes (stars at last). *Trends Neurosci* 2003;26:520–522. [PubMed: 14522143]
- Rash JE, Duffy HS, Dudek FE, Bilhartz BL, Whalen LR, Yasumura T. Grid-mapped freeze-fracture analysis of gap junctions in gray and white matter of adult rat central nervous system, with evidence for a “panglial syncytium” that is not coupled to neurons. *J Comp Neurol* 1997;388:265–269. [PubMed: 9368841]

- Rivieccio MA, John GR, Song X, Suh HS, Zhao Y, Lee SC, Brosnan CF. The cytokine IL-1 β activates IFN response factor 3 in human fetal astrocytes in culture. *J Immunol* 2005;174:3719–3726. [PubMed: 15749911]
- Rouach N, Avignone E, Meme W, Koulakoff A, Venance L, Blomstrand F, Giaume C. Gap junctions and connexin expression in the normal and pathological central nervous system. *Biol Cell* 2002;94:457–475. [PubMed: 12566220]
- Scemes E. Components of astrocytic intercellular calcium signaling. *Mol Neurobiol* 2000;22:167–179. [PubMed: 11414278]
- Sen GC, Sarkar SN. Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev* 2005;16:1–14. [PubMed: 15733829]
- Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;17:1–14. [PubMed: 15585605]
- Theis M, Jauch R, Zhuo L, Speidel D, Wallraff A, Doring B, Frisch C, Sohl G, Teubner B, Euwens C, Steinhauser C, Messing A, Heinemann U, Willecke K. Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43. *J Neurosci* 2003;23:766–776. [PubMed: 12574405]
- Theis M, Speidel D, Willecke K. Astrocyte cultures from conditional connexin43-deficient mice. *Glia* 2004;46:130–141. [PubMed: 15042581]
- Theis M, Sohl G, Eiberger J, Willecke K. Emerging complexities in identify and function of glial connexins. *Trends Neurosci* 2005;28:188–195. [PubMed: 15808353]
- Town T, Jeng D, Alexopoulou L, Tan J, Flavell RA. Microglia Recognize Double-Stranded RNA via TLR3. *J Immunol* 2006;176:3804–3812. [PubMed: 16517751]
- Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 2004;10:1366–1373. [PubMed: 15558055]

Fig. 1.

Microarray analysis of the astrocyte response to pI:C. **(A)** Astrocytes were activated with 10 ug/ml pI:C for 24 h and microarray analysis performed as described in methods. Scatter plots were generated using GenePix Pro software from the normalized microarray data. Data are shown as the mean fluorescence intensity (which is proportional to mRNA expression levels) for each gene on the X-axis plotted against the logarithmic change in expression induced by pI:C above or below the control on the Y-axis. Arrows show data spots for the highly induced gene IL-8 and the potentially down-regulated gene Cx43. **(B)** Astrocytes were cultured in medium alone (0 h timepoint) or activated with 10 mg/ml pI:C for 3, 6, or 24 h and cells harvested for RNA. Q-PCR was performed for IP-10, IL-8, Cx43 and P2X₄R. Data are expressed as fold

increase over the control gene (PBDA) and error bars depict standard deviations obtained from quadruplicate runs. One of two experiments is shown.

Fig. 2.

Poly-[I:C]-induced changes in Cx43 and P2X₄R protein expression. **(A)** Astrocytes were activated with 10 mg/ml pI:C for 3, 6, or 24 h, and cell lysates immunoblotted for Cx43, P2X₄R, and β-tubulin. **(B)**, astrocytes were activated with varying doses of pI:C as shown for 24 h and lysates immunoblotted for Cx43, P2X₄R, and β-tubulin. **(C)** Astrocytes were cultured on MatTek plates with either medium alone or 10 mg/ml pI:C for 24h and immunoreacted for either Cx43 (red, left panels) or P2X₄R (red, right panels), and double-stained for β-tubulin (green) and DAPI (blue). Distance marker is equal to 10 μM.

Fig. 3.

Effect of inhibitors of MEK1/2, JNK, p38 MAP kinase, ERK and PI3kinase on pI:C effects on Cx43 and P2X₄R. Cells were pretreated with inhibitors for 1 h at doses shown, activated with 10 mg/ml pI:C for 24 h or cultured with medium alone. **(A)** Inhibitors used were the MEK1/2 inhibitor UO126; the p38 MAP kinase inhibitors SB203580 and SB202190 and the MEK kinase inhibitor PD098059. Cells were lysed, proteins resolved by SDS PAGE and protein expression determined by immunoblotting for Cx43, P2X₄R, and β-tubulin. **(B)** Cells were treated with the PI3 kinase/Akt inhibitor LY294004 prior to activation with pI:C and lysates immunoblotted for Cx43, P2X₄R, and β-tubulin.

Fig. 4.

Inhibitors of NF- κ B block pI:C-mediated activation of astrocytes. **(A)** Cells were infected with three different doses of an adenoviral construct containing an I κ B α super-repressor (Ad-I κ B) or vector controls (Ad-EV), activated with 10 mg/ml pI:C for 24 h, and protein expression determined by immunoblotting for Cx43, P2X₄R, and β -tubulin. **(A)** A representative blot is shown. **(B)** Cumulative data for three separate experiments are shown. Blots were quantified using Scion NIH Image and values for each protein calculated as a ratio to β -tubulin, and expressed as a percent of the control value, which was set at 100%. Significance values for different treatment protocols are marked with an *, $P < 0.05$. **(C)**, astrocytes were pretreated with the I κ B α inhibitor BAY11-7082, SB203580 or BAY11-7082+SB203580 at doses shown for 1 h, activated with 10 mg/ml pI:C for 24 h, and immunoblotted for Cx43 and β -tubulin.

Fig. 5.

Adenoviral vectors containing a dominant-negative IRF3 block pI:C-mediated activation of astrocytes. (A) Cells were infected with three different doses of an adenoviral construct containing a dominant-negative IRF3 (dnIRF3), or an empty vector control (EV), or wild-type IRF3 (WT), activated with 10 mg/ml pI:C for 24 h, and proteins immunoblotted for Cx43, P2X₄R, P2Y₁, and β -tubulin. (A) A representative blot is shown. (B) cumulative data for three separate experiments are shown. Blots were quantified using Scion NIH Image and values for each protein calculated as a ratio to β -tubulin, and expressed as a percent of the control value which was set at 100%. Significance values are marked with an *, $P < 0.05$.

Fig. 6.

Analysis of pore-formation using YoPro-1. Astrocytes were plated in 96-well black-walled, clear bottom plates in quadruplicate and treated with medium alone as a control (**A**) or 10 mg/ml pI:C (**B**) for 24 h. Cells were cultured in divalent free PBS containing 10 μ M YoPro-1 with or without ATP and BzATP at dilutions shown. Fluorescence levels were determined every 2 min using a SpectraMax Gemini spectrofluorimeter set at 37°C. Data were calculated as percent of the mean time zero value, and the mean value for the quadruplicate wells is shown. All standard deviations were within 10%. One of two independent experiments using astrocytes from two different cases is shown.

Fig. 7.

Analysis of functional gap junction coupling using a scrape loading assay. Astrocytes were plated in 35 mm dishes and either infected with adenoviral vectors containing an IkB SR or a dnIRF3 as described earlier, or pretreated for 1 h with inhibitors. Cells were then treated with 10 ug/ml pI:C or medium alone for 24 h. At the time of testing, cells were washed in divalent ion free PBS pH 7.4, incubated with 0.5 mg/ml Lucifer yellow, scrapes made with a razor, and cells incubated for 10 min. Cells were then fixed and photographed using a FITC filter. An optical density profile of LucY uptake was obtained using Scion NIH Image software. Values of LucY spread were obtained by averaging the maximal distances at which LucY fluorescence could be detected; at least three measurements of LucY spread were obtained along each single scrape line in a minimum of three independent experiments. Fractional changes in LucY spread were calculated as a percent of control. (A) Images of control cells and pI:C-treated cells with

or without pretreatment with adenoviral vectors. **(B)** quantitative analysis of LucY spread for cells that had been pretreated with the dn IRF3 or the I κ B α SR prior to activation with pI:C. **(C)** Quantitative analysis of LucY spread in astrocytes that had been pretreated with the inhibitors BAY11-7082 and/or LY294002 at doses shown prior to activation with pI:C. Significance values for different treatment protocols are *, $P < .001$, **, $P < .0001$, students T test.